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(54) Title: PCR PRIMERS FOR DETECTION OF LEGIONELLA SPECIES AND METHODS FOR CONTROLLING VISUAL INTENSITY IN HYBRIDIZATION ASSAYS

(57) Abstract

This invention provides for superior nucleic acid primers for amplification of select target regions of the genome of the genus *Legionella*. The invention facilitates detection of pathogenic and nonpathogenic forms of this genus. The invention further provides for processes for using the primers in template dependent nucleic acid polymerase extension reactions to amplify select target regions. Kits for the use of these primers are also provided. This invention further provides for methods of controlling the intensity of visual signal for detection of duplex formation in nucleic acid hybridization assays under high stringent conditions. This method involves the blending of different capture probes onto a solid support.

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PCR PRIMERS FOR DETECTION OF LEGIONELLA SPECIES AND METHODS FOR CONTROLLING VISUAL INTENSITY IN HYBRIDIZATION ASSAYS

BACKGROUND OF THE INVENTION

1. Field of the Invention.

This invention provides for superior nucleic acid primers for amplification of select target regions of the genome of the genus *Legionella*. The invention facilitates detection of pathogenic and nonpathogenic forms of this genus. The invention further provides for processes for using the primers in template dependent nucleic acid polymerase extension reactions to amplify select target regions. Kits for the use of these primers are also provided.

This invention further provides for methods of controlling the intensity of visual signal for detection of duplex formation in nucleic acid hybridization assays under high stringent conditions. This method involves the blending of different capture probes onto a solid support.

2. Information Disclosure.

Legionella species are known as both pathogenic and nonpathogenic microorganisms. In the pneumonic form, they are intracellular pathogens of lung macrophage cells. Legionellaceae, Chpt. 9260 J. in Standard Methods of the Examination of Water and Wastewater, Eds. Clesceri, Greenberg and Trussell, 17th Ed. 1989 pages 9-149 to 9-153 and Muraca, P.W. et al., 1988, Environmental Aspects of Legionnaires' Disease, J. Amer. Water Works Assoc. 80:78-86.

A surface antigen of *Legionella* has been implicated as a requirement for intracellular pathogenicity and is called a macrophage infectivity potentiator or *mip*. Cianciotto, N.P. et al., 1989, A *Legionella pneumophila* Gene Encoding a Species-specific Surface Protein Potentiates Initiation of Intracellular Infection, Infection and Immunity, 57:1255-1262.

The nucleotide sequence of the 5S rRNA has been reported by MacDonnell, M.T. and R.R. Colwell, 1987, The Nucleotide Sequence of the 5S rRNA From *Legionella pneumophila*, Nucleic Acid Research, 15:1335; and, by Chumakov, K.M et al., 1986, Use of 5S Ribosomal RNA Nucleotide Sequence Analysis for the Study of Phylogeny of the Genus *Legionella*, Mol. Genet., Mikrobiol Virusol., 8:38-40.

The nucleotide and amino acid sequence of the macrophage infectivity protein is known and reported by Engleberg, N.C. et al., 1989, DNA Sequence of *mip*, a *Legionella pneumophila* Gene Associated With Macrophage Infectivity, Infection and Immunity, 57:1263-1270.

Standard culture techniques for *Legionella* are not adequate to properly assess risks from this deadly pathogen. Hussong, D. et al, 1987, Viable *Legionella pneumophila* Not Detectable by Culture on Agar Media, Bio/Technology 5:947-950.

Nucleic acid probes for detection of *Legionella pneumophila* have been reported. Grimont, P.A.D. et al., 1985, DNA Probe Specific for *Legionella pneumophila*, J. Clin. Micro., 21:431-437; and Engleberg, N.C. et al., 1986, A *Legionella*-Specific DNA Probe Detects Organisms in Lung Tissue Homogenates from Intranasally Inoculated Mice, Israel J. of Med. Sciences, 22:703-705.

The use of polymerase chain reaction amplification methods to detect *Legionella* species has been disclosed by Starnbach, M.N. et al., 1989, Species-Specific Detection of *Legionella pneumophila* in Water by DNA Amplification and Hybridization, J. of Clin. Microbiol., 27:1257-1261; in U.S.S.N. 07/467,813 filed on January 1, 1990; and, also in Detection of *Legionella* in Environmental Samples Using Polymerase Chain Reaction (PCR), Biotechnology Bulletin, May 1990 pages 11-12.

SUMMARY OF THE INVENTION

This invention provides for superior amplification primers for use in assays for detecting pathogenic *Legionella* species. Described primers are able to amplify select regions of the 5 S RNA gene that are uniquely common to most species of

Legionella. Other primers are able to discriminate between *Legionella* having the *mip* gene and those without the *mip* gene. The primers are further advantageous in that they have similar thermal melting points and can be used in combination to efficiently and equally amplify more than one region of the *Legionella* genome in a single amplification reaction mixture.

In particular, this invention provides for a composition comprising multiple pairs of amplification primers for amplifying subsequences of the 5 S RNA gene and *mip* gene of *Legionella* species each primer having a thermal melting point with respect to their binding sites of less than about 8°C of each other. Methods for amplifying genomic regions of *Legionella* using these compositions are also described herein.

This invention further provides for nucleic acid polymerase primers for the amplification of subsequences of nucleic acid from *Legionella* species wherein the primers bind substantially to the nucleic acid subsequence selected from the group consisting of (a) 3'-CGTAACCACGGCTAAACC-5'; Seq. ID No. 12; (b) 3'-CGAAACGGTAGTTTAGAAAGACTT-5'; Seq. ID No. 13; (c) 3'-CCGCTGATATCGCYAAACCTT-5'; Seq. ID No. 14; (d) 3'-CGCTACTGGATGAAAGYGTACT-5'; Seq. ID No. 15; (e) 3'-CCGCTGATATCGCCACACCTT-5'; Seq. ID No. 18; (f) 3'-CGCTACTGGATGAAAGCGTAC-5'; Seq. ID No. 19; and (g) 3'-CAAAACGGTAGTTTAGAAAAACTT-5'; Seq. ID No. 20 where Y represents a cytosine or thymine base and the primers having a base Y encompass a mixture of the two primers. Examples of such primers are: (a) 5'-GCATTGGTGCCGATTTGG-3'; Seq. ID No. 6; (b) 5'-GCTTTGCCATCAAATCTTTCTGAA-3'; Seq. ID No. 7; (c) 5'-GGCGACTATAGCGRTTTGGAA-3'; Seq. ID No. 10; (d) 5'-GCGATGACCTACTTTCRCATGA-3'; Seq. ID No. 9; (e) 5'-GGCGACTATAGCGGTGTGGAA-3'; Seq. ID No. 21; (f) 5'-GCGATGACCTACTTTCGCATG-3'; Seq. ID No. 22; and (g) 5'-GTTTTGCCATCAAATCTTTTTGAA-3'; Seq. ID No. 23 where R represents an adenine or a guanine base and the primers having a base R are a mixture of the two primers.

This invention also provides for an internal positive control oligonucleotide sequence comprising an unnatural oligonucleotide subsequence flanked by primer binding sites.

In this fashion the IPC will amplify with the same primer pairs used to amplify the target subsequence. In one embodiment of the present invention, the target is the *mip* gene of *Legionella* species. In this embodiment the IPC comprises an unnatural subsequence flanked by the following nucleic acid subsequences: (a) 3'-CGTAACCACGGCTAAACC-5'; Seq. ID No. 12; and, (b) 3'-CGAAACGGTAGTTTAGAAAGACTT-5'; Seq. ID No. 13. In another embodiment the primer binding sites of the IPC will be identical to the primer binding sites of the 5 S RNA gene target. In this embodiment the primer binding sites will comprise

(c) 3'-CCGCTGATATCGCYAAACCTT-5'; Seq. ID No. 14; and/or,

(d) 3'-CGCTACTGGATGAAAGYGTACT-5'; Seq. ID No. 15; and/or,

(e) 3'-CCGCTGATATCGCCACACCTT-5'; Seq. ID No. 18; and/or,

(f) 3'-CGCTACTGGATGAAAGCGTAC-5'; Seq. ID No. 19 where Y represents a cytosine or thymine base. More specifically there is disclosed an IPC oligonucleotide sequence having either one or both of the following sequences 5'-GCATTGGTGCCGATTTGG-3'; Seq. ID No. 6 and 5'-TTCAGAAAGATTTGATGGCAAAGC-3'; Seq. ID No. 13.

It should be understood that alternative primers could be envisioned deviating from those described. Deviations might include minor base changes, biotinylated bases, base analogs or additional bases added to the primer ends. Wherever such modifications or changes do not substantially affect the primers' ability to amplify the target region internal to the stated binding sites for the named primers such modified primers are considered to be within the scope of this invention.

This invention further embraces processes utilizing the above amplification primers. Preferably in a multiplex PCR format. In particular there is disclosed a process of using nucleotide polymerases for amplification of nucleic acid subsequences from *Legionella* species wherein the polymerases initiate subsequence amplification by extension of nucleic acid primers which bind substantially to a nucleic acid subsequence selected from the group consisting of: (a) 3'-CGTAACCACGGCTAAACC-5'; Seq. ID No. 12; (b) 3'-

CGAAACGGTAGTTTAGAAAGACTT-5'; Seq. ID No. 13; (c) 3'-CCGCTGATATCGCYAAACCTT-5'; Seq. ID No. 14; (d) 3'-CGCTACTGGATGAAAGYGTACT-5'; Seq. ID No. 15; (e) 3'-CCGCTGATATCGCCACACCTT-5'; Seq. ID No. 18; (f) 3'-CGCTACTGGATGAAAGCGTAC-5'; Seq. ID No. 19; and (g) 3'-CAAAACGGTAGTTTAGAAAAACTT-5'; Seq. ID No. 20 where Y represents a cytosine or thymine base and the primers having a base Y are a mixture of the two primers. This invention also provides for the above process where the primers are as described above and where an internal positive control oligonucleotide sequence is included in the amplification mixture.

This invention provides for kits for the amplification of nucleic acid from *Legionella* species comprising a compartment which contains a nucleic acid which binds substantially to a nucleic acid subsequence as described above and preferably containing the primers as described above. Internal positive control oligonucleotide sequence are also included in an alternative embodiment of these kits.

A second aspect of the disclosed invention relates to controlling the intensity of the visual signal used to detect duplex formation in nucleic acid hybridization assays. The model is a nucleic acid hybridization assay kit for the detection of a particular genus having numerous distinct species. In some cases detection and discrimination of a particular species may be desirable especially where a particular species is known to be of medical importance. In other cases where most of the species of organisms within a genus have been associated with disease, it is desirable to detect the presence the entire genus of organisms. It is further desirable that the detection of the genus of organisms be obtained as one positive visual response regardless of species. If the target nucleic acid sequence used for the detection of the genus of organisms varies for different species within the genus it is typically necessary to lower the stringency of hybridization or utilize multiple capture probes. This invention avoids this problem of resorting to low stringent conditions.

Lower stringent conditions are routinely used to accommodate the capture of multiple target sequences that contain variations in their nucleic acid sequences. The stringency is reduced by either lowering the temperature of hybridization and wash or by modification of the buffer. When the stringent conditions are reduced and the target nucleic acid sequence is very similar to nucleic acid sequences of another genus of organisms specificity of the capture probe for the target genus can be lost.

When multiple capture probes are used and are selected to be compatible to variations in the target nucleic acid sequences, the specificity under high stringent conditions can be regained. The blending of multiple probes permits a single positive response for the presence of a group of target organisms. Without this invention, the different capture probes would normally be immobilized individually on a solid support. As a result, the assay would require the use of more test sample, more time to perform the test and more interpretation by the user.

In those kits utilizing this invention, the multiple capture probes are blended together and immobilized in such a manner to accommodate the detection of the various species of the genus of organisms in a single test. The probes are selected to be compatible to blending (i.e. contain noncomplementary sequence and have as similar as possible melting temperatures). The capture probes should be blended in the proper ratio to generate a result of equal intensity for species within the genus of organisms to minimize interpretation of the result when semiquantitation and equal sensitivity for all target organisms are desirable. Depending on the hybridization characteristics, the concentration each capture probe in the blended may or may not be equal. It is possible to determine the proper capture probe blend by a series of titrations. The blends of the capture probes of varying ratios are created and immobilized on a solid support.

The capture probes are individually immobilized on the solid support for reference in the titration of the probe blend. Nucleic acid from different species within the genus of

organisms to be tested are hybridized to the capture probe blends and to the individual probes. The probe blend that generates the same response as an individual specific probe for the species within the genus is chosen for use as a capture probe blend in the kit. These capture probes may also be individually be immobilized as capture probes should further speciation be desired but it is not necessary.

It should be noted that the mixture of capture probes may or may not contain all probes for the same gene provided that they have similar hybridization characteristics.

The mixture of capture probes may be able to capture more than one genus if desired. For example, should a kit be developed that detects food borne pathologies such as the genus *Salmonella* and *Shigella*, a mixture of probes can be used to indicate a pathogen is present of either of the two genera. This may be all that is necessary for the food laboratory. If further identification is necessary (maybe for food poisoning problem) then the same capture probes may be used for identification in separate and unblended states.

More specifically, this invention provides for a method for controlling visual density of detection probes bound to capture probes in a nucleic acid hybridization assay having a first and a second capture probe wherein the nucleic acid sequences of the two capture probes are different, said method comprising: (a) mixing a blend of the two different capture probes; (b) affixing the mixture of probes from step (a) and the first capture probe to discrete first and second regions of a solid support wherein the concentration of the first capture probe is equal in both regions; (c) binding the detection probes to the capture probes of step (b) under high stringent hybridization conditions such that the detection probes bind to one capture probe but not the other; and (d) detecting hybridization of the detection probe by visual density; wherein the proportion of the two capture probes in the blend of step (a) is adjusted to give a visual density in the first region equal to that of the second region having the first capture probe when equal amounts of the detection probes are present in a test sample. The concentration of the second probe in the

second region will not be the same as the concentration of the second probe in the first region. Typically the second probe is not present in the second region.

The detection probe can be an amplification product such as a PCR amplification product. The assay format can involve a detection probe which is a complex of a target nucleic acid which binds under high stringent hybridization conditions to different regions of the capture and signal probe and wherein the capture and signal probes do not hybridize to each other.

Furthermore it is preferred that this method deliberately provide for a proportion of the two capture probes adjusted according to a known quantity of target nucleic acid. The detection range for the assay is in a concentration range of between about 10×10^{-12} to about 100×10^{-9} molar. It being desired that the assay provide a positive gradient of visual density over a target nucleic acid concentration of between about 10×10^{-12} to about 100×10^{-9} molar.

Kits embracing the above method are also a part of this invention. In particular there is contemplated a nucleic acid hybridization kit providing equal visual density for positive results in a test sample. The kit uses detection probes and a first and a second capture probe wherein the capture probes have different nucleic acid sequences, said kit comprising the following components: (a) a solid support having a first discrete region having a concentration of the first and second capture probes affixed thereto; (b) a solid support having a second discrete region wherein the first capture probes are affixed thereto in a concentration equal to the concentration of the first probe in the mixture described in component (a); and, (c) a container containing detection probes; wherein the proportion of the two capture probes in the mixture of component (a) is adjusted to give a visual density equal to that of the solid support of component (b) under identical hybridization conditions and with equal amounts of the detection probes present in the test sample. The concentration of second probe in the second region is not the

same as its concentration in the first region and is typically not present.

DEFINITIONS

"Affixing" in the context of nucleic acids and solid supports refers to the binding or attaching of the nucleic acids to solid supports by conventional means such as covalent or electrostatic bonding.

"Amplification reaction mixture" refers to an aqueous solution comprising the various reagents used to amplify a target nucleic acid. These include enzymes, aqueous buffers, salts, target nucleic acid, and nucleoside triphosphates. Depending upon the context, the mixture can be either a complete or incomplete amplification reaction mixture.

"Amplification reaction system" refers to any *in vitro* means for multiplying the copies of a target sequence of nucleic acid. Such methods include but are not limited to PCR, DNA ligase, QB RNA replicase and RNA transcription-based amplification systems. These involve multiple amplification reagents and are more fully described below.

"Amplification reaction tube(s)" refers to container suitable for holding the amplification reagents. Generally the tube is constructed of inert components so as to not inhibit or interfere with the amplification system being used. Where the system requires thermal cycling of repeated heating and cooling, the tube must be able to withstand the cycling process and typically precisely fit the wells of the thermocycler.

"Amplification reagents" refer to the various buffers, enzymes, primers, nucleoside triphosphates both conventional and unconventional, and probes used to perform the selected amplification procedure.

"Amplifying" or "Amplification" which typically refers to an "exponential" increase in target nucleic acid is being used herein to describe both linear and exponential increases in the numbers of a select target sequence of nucleic acid.

"Bind(s) substantially" refers to complementary hybridization between oligonucleotides and embraces minor

mismatches which can be accommodated by reducing the stringency of the hybridization media to achieve the desire priming of the PCR polymerases.

"Biotinylated" refers to a biotin moiety covalently attached to the 5' end of an oligonucleotide for the purpose of reacting with streptavidin in a detection assay.

"Bound" in the context of nucleic acids refers to the hybridization of the nucleic acids through complementary base pairing.

"Capture probes" refer to oligonucleotides which are bound to a solid support and are capable of hybridizing with detection probes either directly or indirectly through target nucleic acid. The capture probes may be bound to the solid support through a variety of known means including covalent bonds and electrostatic bonds.

"Concentration" refers to the molar amount of a substance in a given amount of volume or space.

"Detection probes" refers to nucleic acids which bind to capture probes. The detection probe either is directly detectable as with a radioisotope incorporated into the sequence or indirectly labelled as with biotin wherein a streptavidin/enzyme complex is subsequently bound. Detection probe is also meant to include a target nucleic acid/signal probe complex of a ternary or quaternary sandwich hybridization assay. Such systems typically require the target nucleic acid to bind to the capture probe and the signal probe then binds to the target nucleic acid.

"Discrete" refers to regions of capture nucleic acid affixed to the solid support having boundaries which provide separation and identification allowing one to distinguish between other regions of capture nucleic acid.

"High stringent hybridization conditions" refers to temperature, solute concentrations and polarity of a given hybridization medium which provides detectable differences in hybridization rates between oligonucleotides of between 10 and 30 bases wherein the oligonucleotides mismatch their respective complementary bases by 1 mismatched base pair. The different

hybridization rates are distinguished using the detection system of the given assay.

"Internal positive control (IPC) oligonucleotide sequence" refers to a recombinant or synthetic oligonucleotide that amplifies with the same primer pair used to amplify target nucleic acid. IPC oligonucleotide sequences are used to ensure assay users that the amplification process has occurred in the event that the sample being tested has no target nucleic acid. These oligonucleotides are flanked at one or both ends by a sequence complementary to the binding site of an amplification primer used in the amplification process. The internal target subsequence is a foreign sequence not naturally found adjacent to the binding sites of the amplification primers. The internal control oligonucleotide sequences thus serve as templates for the amplification primers to establish that the amplification reaction would have amplified target nucleic acid if such target had been present. IPC oligonucleotide sequences can be supplied as single or double stranded nucleotides. When single stranded those of skill would recognize that to facilitate non-linear amplification the 5' end of the IPC should have base identity with the other primer of the pair.

"*Legionella* species" include those members of the family Legionellaceae. It is one genus family composed of more than 22 species. It should be recognized that the taxonomy of prokaryotes is not static and that additional species might be named or that these species may some day be incorporated into an alternative family or genus.

"Nucleic acid" refers to a deoxyribonucleotide or ribonucleotide polymer in either single or double stranded form and unless otherwise limited would encompass known analog of natural nucleotides which can function in a similar manner as naturally occurring nucleotides.

"Nucleotide polymerases" refers to enzymes able to catalyze the synthesis of DNA or RNA from nucleoside triphosphate precursors. In the amplification reactions of this invention the polymerases are template dependent and typically extend from the 3' end of the polymer being formed. It is most preferred that the polymerase is thermostable as

described in U.S. Patent No. 4,889,819 and U.S. Serial No. 7/143,141, filed January 12, 1988, now abandoned.

"Positive gradient of visual density" refers to a response of visual density which increases with the presence of an increased presence of detection probe. In the assays described herein it is possible to provide a dynamic range of concentrations wherein an increase in detection probe is noticeable. Below this range, there is little or no visible density and above the range, increased detection probe in a sample will not yield increased visual density.

"Primer" or "nucleic acid polymerase primer(s)" refers to an oligonucleotide, whether natural or synthetic, capable of acting as a point of initiation of DNA synthesis under conditions in which synthesis of a primer extension product complementary to a nucleic acid strand is initiated, i.e., in the presence of four different nucleotide triphosphates and an agent for polymerization (i.e., DNA polymerase or reverse transcriptase) in an appropriate buffer and at a suitable temperature. A primer is preferably an oligodeoxyribonucleotide and is single stranded for maximum efficiency in amplification, but may also be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. The exact length of a primer will depend on many factors, but typically ranges from 15 to 25 nucleotides. Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with the template. A primer need not reflect the exact sequence of the template but must be sufficiently complementary to hybridize with a template. An example of a non-complementary sequence which may be incorporated into the primer is a sequence which encodes a restriction enzyme recognition site (see U.S. Patent No. 4,800,159).

A primer can be labeled, if desired, by incorporating a label detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include ^{32}P, fluorescent dyes, electron-dense reagents, enzymes (as commonly used in ELISAS), biotin, or

haptens or proteins for which antisera or monoclonal antibodies are available. A label can also be used to "capture" the primer, so as to facilitate the immobilization of either the primer or amplified DNA on a solid support.

"Proportion of the two capture probes in the blend" refers to the relative concentrations of the probes, (i.e., 1:1 or 2:1).

"Signal probe" refers to an oligonucleotide which comprises a detectable label. This term is generally used to differentiated between the target nucleic acid and capture probe in a ternary or quaternary sandwich assay format. The label can be any of the reporter or signal systems described for the detection probe.

"Solid support" refers to any insoluble material which can provide a substrate upon which to affix capture nucleic acids. Such substrates may include nylon, amino or carboxy activated plastics, glass, cellulose and the like.

"Subsequence" refers to a sequence of nucleic acids which comprises a part of a longer sequence of nucleic acids.

"Target region" refers to a region of a nucleic acid which is to be analyzed, which usually contains polymorphic DNA sequences.

"Target nucleic acid" refers to a nucleic acid which complexes with a signal probe to provide the detection probe. Such nucleic acids are typically used in the sandwich assay formats wherein the target nucleic acid binds to a capture probe and the signal probe binds to the target nucleic acid.

"Test Sample" refers to an aqueous hybridization medium containing detection probes.

"Uniform density" refers to equivalent density either through visual recording with the eye or with an analytic instrument such as a densitometer or colorimeter.

"Visual density" refers to the relative intensity of the signal or detection product produced by the labelling system used to measure the presence or absence of duplex formation between complementary oligonucleotides. The choice of labelling systems is not critical. The system can be direct or indirect and may be radioactive, fluorescent, luminescent or

colorimetric. Density may be determined either by photographic means, analytic instruments or by the naked eye. Enzymatic colorimetric labeling systems are preferred.

DETAILED DESCRIPTION

Introduction:

The detection of *Legionella* species using PCR to amplify nucleic acid from these species requires multiple steps. These steps include an adequate sampling procedure to isolate and/or concentrate the extant organisms to a suitable degree for detection, a method for lysing the cells to release nucleic acid, a suitable procedure to amplify the target nucleic acid sequences and a means to detect the amplified sequences. A general description of this methodology can be found in Atlas, R.M. and Bej, A.K., Detecting Bacterial Pathogens in Environmental Water Samples by Using PCR and Gene Probes in PCR Protocols, Ed. Innis et al., Academic Press, Inc. 1990 at pages 399-406.

To effectively amplify target nucleic acid subsequences, PCR requires primers to initiate polymerase extension. The selection of primers is an important aspect of this invention and the particular primers disclosed herein solve various problems associated with other primers purported to function adequately for the detection of *Legionella* species. Such problems relate to the binding of primers to hairpin turns within the genome, undue internal secondary structure within primers themselves, non-specific binding of the primers to both target regions and to non-target regions, unequal thermal melting points such that the primers are not all efficiently binding at the same temperature and maintaining relatively short amplified product.

The 5S RNA gene amplification primers of this invention are able to amplify numerous species of the *Legionella* genus (see Table 1). They advantageously do not amplify closely related species from the genus *Pseudomonas* or *Flavobacterium*. The *mip* primers are specific for their ability to amplify the *mip* gene of *L. pneumophila*, *L. hackeliae* and strains of *L. sainthelensi*.

Sampling systems:

Legionella exists in warm aquatic environments. Human infections of pathogenic *Legionella* often involve aerosol exposure to warm fresh water such as that found in air conditioning systems and residential water supplies. For purposes of sampling, suspect water is collected and either subjected to low speed centrifugation (eg., 10,000 X g for 15 minutes) or filtered to concentrate or collect the *Legionella*. *Legionella* are trapped by filters having a porosity of about 0.45μm. A prefilteration step can be useful where physical debris may interfere with the subsequent filtration or amplification procedures. The prefilter is a noncritical feature of this invention. Such filters are generally of a porosity and composition that will allow the *Legionella* bacterium to pass freely through it. The physical means for water collection are well known and include sterile glass vials, syringe assemblies and other inert vessels suitable for storage of water.

Sample preparation:

Relatively clean water samples may be directly assayed without isolation or purification of the target nucleic acid prior to amplification. Large water samples may be filtered to concentrate microbial populations. Filtering procedures and filters are well-known to those of skill. See, for example, Standard Method For The Examination Of Water and Wastewater, 17th Ed., page 9-14, published by the Amer. Publ. Health Assoc., 1015 Fifteenth St. N.W., Washington, D.C. 20005. Preferred filters have a porosity which will trap *Legionella* cells yet permit extraneous nucleic acid to pass. Teflon membranes such as Fluoropore FGLP 0013 of 0.2 μM, or 0.5 μM pore size (Millipore Corp., Bedford, MD), or Duropore HVLP are of use in this invention.

The water samples or filter concentrated samples are subjected to conditions sufficient to release the target nucleic acid from the suspect organisms. Mechanical lysis is preferred. Mechanical lysis can be achieved by sonication, boiling or multiple freeze/thaw cycles. Boiling the sample in

the presence of Chelex®, resin is preferred. Chemical means of cell disruption are also operable and include standard lysing means such as lysozymes, osmotic shock, protease K treatment, and detergents. Chemical methods are less preferred because of possible detrimental effects on the PCR process, (ie. *inhibition of the Taq polymerase*).

The samples can be heated to denature proteases and nucleases which might interfere with the components of the PCR reaction mixture. Heating the samples to 85°C for about 5 minutes is generally sufficient. Alternatively, chemical nuclease and protease inhibitors can be used.

When the sample is a complex mixture such as from a patient suspected of being infected with *Legionella*, it may be preferable to isolate the nucleic acid from the original sample. A variety of techniques for extracting nucleic acids from biological samples are known in the art. For example, see those described in Higuchi, "Simple and Rapid Preparation of Samples for PCR" chapter 4 in PCR Technology ed. Erlich, Stockton Press 1989); Maniatis *et al.*, 1989, Molecular Cloning: A Laboratory Manual, (New York, Cold Spring Harbor Laboratory, 1982; Hagelberg and Sykes, 1989, Nature 342:485; or Arrand, *Preparation of Nucleic Acid Probes in Nucleic Acid Hybridization*, A Practical Approach, Ed Hames and Higgins, IRL Press. pp. 18-30, 1985. Whole nucleic acid extraction procedures typically involve an initial contacting with phenol, phenol/chloroform or guanidinium salts, followed by an alcohol precipitation. Genomic DNA may be obtained from a whole nucleic acid extraction by using Rnase before further alcohol precipitation.

PCR procedures:

Although the PCR process is well known in the art (see U.S. Patent Nos. 4,683,195 and 4,683,202 which are incorporated herein by reference) and although a variety of commercial vendors, such as Perkin-Elmer or Perkin-Elmer Cetus instruments, sell PCR reagents and publish PCR protocols, some general PCR information is provided below for purposes of

clarity and full understanding of the invention to those unfamiliar with the PCR process.

To begin the PCR process, the target nucleic acid in the sample is denatured (assuming the sample nucleic acid is double-stranded). Denaturation is typically achieved by heating the samples. This is because chemical denaturants may inhibit the polymerase activity.

Once the strands are separated, the next step in PCR involves hybridizing the separated strands with primers that flank the target region or subsequence. The primers are then extended to form complementary copies of the target strands, and the cycle of denaturation, hybridization, and extension is repeated as many times as necessary to obtain the desired amount of amplified nucleic acid.

Template-dependent extension of primers in PCR is catalyzed by a polymerizing agent in the presence of adequate amounts of four deoxyribonucleotide triphosphates (dATP, dGTP, dCTP, and dTTP) in a reaction medium comprised of the appropriate salts, metal cations, and pH buffering system. Suitable polymerizing agents are enzymes known to catalyze template-dependent DNA synthesis. For example, if the template is RNA, a suitable polymerizing agent to convert the RNA into a complementary DNA (CDNA) sequence is reverse transcriptase (RT), such as avian myeloblastosis virus RT. Once the target for amplification is DNA, suitable polymerases include, for example, *E. coli* DNA polymerase I or its Klenow fragment, T_4 DNA polymerase, and *Taq* polymerase, a heat stable DNA polymerase isolated from *Thermus aquaticus* and commercially available from PECI. The latter enzyme, *Taq* DNA polymerase, is widely used in the amplification and sequencing of nucleic acids. The reaction conditions for using DNA polymerases are known in the art, and are described in, for example, the treatise Methods in Enzymology, and in Maniatis *et al.*, Molecular Cloning: A Laboratory Manual, *supra*.

During the PCR process, the temperature is very carefully controlled so that strand separation and primer annealing and extension occur in equilibrium. The control of

temperature is typically achieved using dry heat generated from a thermocycler.

In the preferred embodiment of the PCR process, the reaction is catalyzed by a thermostable DNA polymerase enzyme, such as *Taq* DNA polymerase, and carried out at an elevated temperature. The preferred temperature is one at which the enzyme is thermostable, and at which the nucleic acids are in an equilibrium of single and double strands, so that sufficient primer will anneal to template strands to allow a reasonable rate of polymerization. Strand separation is achieved by heating the reaction to a sufficiently high temperature for sufficient time to cause the denaturation of the duplex, but not to cause an irreversible denaturation of the polymerase.

The PCR method can be performed in a step-wise fashion, where after each step new reagents are added, or in a fashion where all of the reagents are added after a given number of steps. For example, if strand separation is induced by heat, and the polymerase is heat-sensitive, then the polymerase will have to be added after every round of strand separation. However, if, for example, a helicase is used for denaturation, or if a thermostable polymerase is used for extension, then all of the reagents may be added initially, or, alternatively, if molar ratios of reagents are of consequence to the reaction, the reagents may be replenished periodically as they are depleted by the synthetic reaction.

Those skilled in the art will know that the PCR process is most usually carried out as an automated process with a thermostable enzyme. In this process, the reaction mixture is cycled through a denaturing step, a primer annealing step, and a extension step. A DNA thermocycler, a machine specifically adapted for use with a thermostable enzyme is disclosed more completely in EP 236,069 and U.S. Patent No. 4,889,819. DNA Thermocyclers are commercially available from Perkin-Elmer or Perkin Elmer Cetus Instruments (PECI), Norwalk, Conn.

A preferred mode for carrying out the PCR reaction is the multiplex mode. The multiplex mode involves the simultaneous amplification of different target regions using

more than one set of PCR primer pairs. The multiplex procedure is preferably designed around primer pairs which have similar thermal melting points. It is preferred that all pairs have Tms within 8° of each other and that the average Tm is between 45 and about 70°C with preference for an average Tm of between 60° and 70°C.

Selection of the *Legionella* specific primers:

The primers of this invention are provided in Table 2. The primer pairs responsible for amplifying the 5S rRNA of *Legionella* species are a degenerate mixture of primers. This mixture is valuable for ensuring that only target DNA from *Legionella* 5S RNA is amplified. By comparison and empirical analysis it was determined that the left and right primers for the 5S RNA will encompass at least five species of *Legionella* including the important pathogenic forms. These include *L. pneumophila*, isolates Bloomington, Knoxville -1, Philadelphia-1 and Togus-1), *L. pneumophila* fraseri (Los Angeles), *L. bozemanii* and *L. dumoffii*, *L. jordanis*, *L. gormanii*, *L. longbeachae* and will not react to the DNA of common bacteria present in water samples, ie., *Flavobacterium rigense*, *Pseudomonas fluorescens* and *aerogenes*, *Proteus vulgaris*, and *Escherichia coli*. Table 1 provides a list of *Legionella* species able to be detected using the disclosed 5S RNA primers. These primers also have compatible Tms of about 65°C and bind to regions of target sequences having relatively low secondary structure.

The left and right primers for the *mip* gene are directed to amplify that region of the gene which is specific for pathogenicity and able to identify the species *L. pneumophila* from most other *Legionella* species. Both these primers have Tms of about 68°C and bind to highly conserved regions of the *Legionella* genome that have little tendency to form secondary structures such as hairpins,

Table 1. *Legionella* species having target 5 S RNA genes amplified by PT82 and PT80

1. *Legionella pneumophilia* SG4

2. *L. pneumophilia* SG3
3. *L. pneumophila* SG11
4. *L. pneumophila* SG6
5. *L. pneumophila* SG7
6. *L. pneumophila* SG10
7. *L. longbeachae* SG1
8. *L. pneumophila* SG2
9. *L. hackeliae* SG1
10. *L. pneumphilia* SG1
11. *L. feeleii* SG2
12. *L. pneumophila* SG9
13. *L. erythra*
14. *L. jordanis*
15. *L. pneumophila* SG15
16. *L. tucsonensis*
17. *L. pneumophila* SG12
18. *L. dumoffii*
19. *L. pneumophila* SG8
20. *L. jamestowniensis*
21. *L. spiritensis*
22. *L. sainthelensi* SG1
23. *L. maceachernii*
24. *L. moravica*
25. *L. quinlivanii*
26. *L. cincinnatienisis*
27. *L. bozemanii* SG1
28. *L. parisiensis*
29. *L. feeleii* SG1
30. *L. parisiensis*
31. *L. feeleii* SG1
32. *L. santicrucis*
33. *L. pneumophila* SG5
34. *L. birminghamensis*
35. *L. cherrii*
36. *L. bozmannii* SG2
37. *L. micdadei*
38. *L. oakridgensis*
39. *L. gratiana*

40. *L. pneumophila* SG14
41. *L. anisa*
42. *L. pneumophila* SG13
43. *L. gormanii*
44. *L. wadsorthii*
45. *L. longbeachae* SG2
46. *L. sainthelensi* SG2

Detection of amplified product:

The detection of the PCR products can be accomplished by direct visualization of the gels following ethidium bromide staining or by indirect means using specific nucleic acid hybridization probes. These methods are well known to those of skill and a general review of such techniques can be found in Nucleic Acid Hybridization, A Practical Approach, Eds. Hames and Higgins, IRL Press, Washington, D.C. 1985.

When detection of the PCR products is by hybridization as in a Southern blot, nucleic acid probes specifically complementary to a subsequence of the amplified region are used. Such probes are readily obtainable from the sequence of the amplified segment. Probes preferably hybridize to a DNA subsequence located between the primer binding subsequences to avoid any overlap of primer sequences and probe sequence. Preferred probes for the 5S rRNA and the *mip* gene of *Legionella* are provided in Table 2 (capture probes).

Depending on the assay format, it may be helpful to have labelled probes function to detect the amplified product. The probes can be labeled by any of the methods known in the art. Radioactive and enzyme labels are preferred. Most preferred are enzyme labels which in the presence of an appropriate substrate will produce a colored product. Examples include horseradish peroxidase and alkaline phosphatase. Color development can be accomplished by a variety of means using a variety of known substrates. Preferred methods for horseradish peroxidase include using tetramethylbenzidine (TMB) as described in Clin. Chem. 33(8):1368-1371 (1987). An alternative detection system is the Enhanced Chemiluminescent

(ECL) detection kit commercially available from Amersham. The kit is used in accordance with the manufacturer's directions.

The electrophoresing conditions and the means for detecting the individual amplified oligonucleotides are well known and are not critical aspects of this invention. Any of the means accepted by those of skill will be applicable for this invention.

An alternative mode of detection of the amplified product is by sandwich hybridization onto membrane strips. In the typical arrangement, the strip is made of nylon and has oligonucleotide capture probes covalently bound in discrete spots.

To bind oligonucleotide capture probes to nylon, they are first tailed with oligothymidylic acid of about 100 bases. Tailing can be achieved using terminal transferase such as calf-thymus terminal deoxynucleotidyl transferase or polythymidine oligonucleotides can be chemically synthesized along with the probes. The tailed oligonucleotides are spotted onto the nylon strips. The strips are dried overnight and exposed to ultraviolet irradiation. The strips are then stored until hybridization assays are conducted. Additional details are provided in copending and co-assigned U.S. patent application, Serial No. 347,495, filed May 4, 1989 is hereby incorporated by reference.

Capture probes are as described in tables 2 and 3 and are able to bind to the amplified products of the *Legionella* genome. Amplified product is allowed to bind to the membrane through hybridization to the capture probes. Amplified product is then detected by enzyme activity. A preferred means for detection of amplified product is to biotinylate each primer so that the amplified product is able to bind a streptavidin-horseradish peroxidase complex. The complex then permits detection of amplified product by chromagen development using commercially available substrates. Primer pairs may be biotinylated using the procedures described in Levenson C. and C. Chang, Nonisotypically Labeled Probes and Primers in PCR Protocols, Ed. Innis et al., Academic Press, Inc. 1990 at pages 99-112. Alternatively synthesis using a phosphoramidite

linkage to directly biotinylate is available using reagents from Glenn Research.

Kits:

The primers of this invention can be embodied into kits for the detecting *Legionella* species in the environment. Such kits would include a variety of components. The kits would preferably include *Legionella pneumophila* DNA preferably a known pathogenic form such as the Bloomington isolate. This DNA will serve as a positive control. A bulk polymerase chain reaction mix which would contain optimized concentrations of Amplitaq® DNA Polymerase, a thermal resistant nucleic acid polymerase available from PECI, all 5S rRNA primers, all *mip* primers, dNTPs (dUTP preferably replacing dTTP), and buffer salts. An internal positive control sequence (IPC) is included to ensure that the PCR procedure functions properly. The IPC comprises an amplifiable target which acts as a positive control regardless of whether the *Legionella* target nucleic acid is present. In a preferred embodiment, the IPC comprises an unnatural sequence selected from a portion of the human HLA DQα gene. The gene sequences are flanked by sequences complementary to the upstream and downstream primers used to amplify subsequences of either the *mip* or 5 S RNA genes. Human HLA DQα is a sequence which is relatively rare in water samples and not likely to be present in the sample at a detectable level. The sequence for a preferred IPC is provided in Table 3. The IPC can be either double or single stranded and may be stored in the amplification reaction mixture or in a separate container. Typically, IPC is added to an amplification reaction mixture at 1 million to 10 million copies. For quantitation of *Legionella* present in the samples, one can use lower amounts of IPC, e.g., 100 to 100,000 copies. A 25 mM $MgCl_2$ solution is included to start the reaction by activation of Taq polymerase.

The kit may also comprise a means to detect the amplified product. Although any standard detection means can be used, such as a hybridization dot blot, agarose, or acrylamide gel electrophoresis, a preferred method uses a dip

stick having a capture oligonucleotide which binds specifically to the amplified target. The dip stick captures the target and is detected by a non-isotopic detection system. For example the primers may be biotinylated before extension and a horseradish peroxidase/streptavidin complex used to bind the enzyme specifically to the capture site of the amplified target. A suitable substrate\buffer combination is 3, 3', 5, 5' tetra methylbenzidine (TMB) in 100 mM Sodium Citrate at pH 5.0.

Controlling visual density of nucleic acid hybridization assays:

In developing commercial quality kits, it is preferable to control the visual density of the detection system. Visual density conveys positive or negative results to the user and are important features of any commercial kit. It is the purpose of the present invention to provide a uniform and controllable visual density for such assays.

The basic problem arises from the desire to provide clear positive/negative signals when using stringent hybridization conditions. Nucleic acid hybridization assays attempting to distinguish between a defined group and less than all the group may benefit by placing in one site of a solid support, a mixture of the capture probes which individually could not capture all the different detection probes representing the group. Such assays will typically have a situs on a solid support bearing the mixture and other sites having less than all the different capture probes affixed thereto.

Without this invention, nucleic acid hybridization assays seeking to identify and distinguish between a group and less than all the group would not provide uniform density of detection when assaying samples containing similar concentrations of detection probes representing the entire group, a subgroup or different subgroups. The reason for the difference in visual density arises from the difference in the thermal melting temperatures [Tm] of the various nucleic acids comprising the assays. This problem becomes particularly acute

in groups where the distinction between members can only be made by distinguishing between oligonucleotides having relatively few base differences, e.g. 1-5 base differences in a 30 base region. Such situations require stringent hybridization conditions and thus differences in thermal melting temperatures, not otherwise a problem, will lead to sharp distinctions between visual densities of positive results where equal concentrations of detection probes are present.

In brief this method provides for a method for controlling visual density of detection probes bound to capture probes in a nucleic acid hybridization assay. The assays typically have two or more different capture probes fixed to a solid support.

The capture probes are mixed in one site of the support according to an empirically determined proportion. A second site containing less than all the capture probes also prepared wherein the concentration of capture probe is approximately equal to the concentration of that probe present in the mixture. This proportion is designed to provide a uniform intensity of visual density between the two sites under a given hybridization condition and given detection probe concentration. The goal being to provide under stringent conditions a uniform density of label or uniform concentration of detection probes between the sites despite the differences in Tm between capture probes.

To assist in the understanding of this invention, a generic model is presented. The model is a nucleic acid hybridization assay kit for the detection of both pathogenic species in Genus A and for detection of the two species individually. The target nucleic acid will be a twenty base region of the 5S ribosomal RNA. This region will differ between the two species in that one species has a guanosine:cytosine instead of an adenosine:thymine pairing. Thus the Tm for species having the extra G:C pair is higher than for the other species.

The assay is as described herein for the *Legionella* assays. A collection of capture probes are first affixed to a solid support and PCR amplification product having

biotin/streptavidin bound enzyme labels are used as detection probes. The array of capture probes on the solid support are three: (1) a first situs containing a mixture of 1:1 of the species specific capture probes to identify, none, either or both members of the genus; (2) a second situs containing only capture probes specific for species 1; and (3) a third situs containing only capture probes specific for species 2.

If the mixture of situs 1 is a 1:1 mixture, the density of the detection probes hybridized thereto for a given concentration of probes to in a test sample will provide density X+Y where X' and Y' are the relative proportion of detection probes specific to species 1 and 2 in a sample. The problem is that density X will not necessarily begin to equal Y where concentration of X' and Y' begin to equal each other in a sample. This is because under high stringent hybridization conditions, the numbers of detection probes in a aqueous sample that bind to capture probes will be different according to their respective thermal melting points.

One solution is to dilute the capture probe having the higher Tm to accommodate the capture probe having a lower Tm. For example in the above generic model, it is suggested that the mixture of capture probes to species 1 and 2 be diluted 1:1, 2:1, 1:2, 3:1 and 1:3. The other sites containing only one capture probe will be likewise diluted. A reference solution of detection probes at a given concentration is then used along with various dilutions to achieve a rough estimate of the visual density of the system for a given hybridization solution and detection/labelling system.

By further routine titration, it is possible to achieve a multiple dot nucleic acid hybridization system using highly stringent hybridization conditions where the visual density of the results from the detection system are equal for a given concentration of detection probe regardless of the Tms for the individual oligonucleotides. Such a system will have a mixture of capture probes in a given proportion affixed to a solid support and a concentration of individual capture probes affixed to another solid support or different region of the same solid support where the individual capture probes are

present on the solid support in concentrations equivalent or approximately equivalent to their concentration in the mixture. It being further understood that the invention is equally applicable to situations where the mixture of probes are more complex than two members and the individual capture probes may represent multiple capture probes that are less than the entire group.

In the preferred assay format, the detection probe will be an amplification product wherein PCR is used to amplify a select target region of an organism. The PCR process yields biotinylated amplification products which serve as detection probes. These assays use a reference number of original copies in a given sample and the sensitivity of the assays is adjusted accordingly. For example, if a regulatory agency wants a detection level of 100 cells per liter and each cell has 10 copies of a target region, the assays can be designed to a collect one liter of sample and extract the nucleic acid from the collected cells. The 1000 targets are then amplified by 30 rounds of PCR at 95% efficiency to yield $5x10^{11}$ copies. This number is then converted into moles using Avogadro number.

The sample of $5x10^{11}$ copies represents the minimum acceptable sensitivity. The sample is then used as a reference against other cells in the group (at similar concentrations) to define conditions yielding an acceptable visual density for that concentration.

This process defines the dynamic range of an assay. The dynamic range are those concentrations of detection probes wherein the subsequent visual density is detectable and directly responsive to increases in the concentration of the detection probe. The dynamic range is defined empirically. It is simply a matter of routine titration to set out acceptable hybridization conditions and labelling systems to provide the desired dynamic range. For the colorimetric system described in this invention using TmB, the preferred dynamic range is between 10×10^{-12} to about 100×10^{-9} molar.

This invention has broad application to the field of nucleic acid hybridization. The means for affixing nucleic acids to solid supports, to detecting hybridized nucleic acid,

to isolating, selecting and amplifying probes, and for formatting the assay including sandwich assays of three or more oligonucleotides and binary assays of two oligonucleotides are well known in the art. These features are not critical to the invention. For an overview of the underlying technology, see U.S. Pat. Nos. 5,015,569 and 4,886,741. A primer on this technology is Nucleic Acid Hybridization, A Practical Approach, Eds. Hames, B.D. and Higgins, S.J., ILR Press 1987. These three references are incorporated herein by reference.

An example of use of blended capture probes for development of a *Legionella* test kit is described in the Example section below. A target sequence of the 5S rRNA gene was used. The 5S RRNA nucleic acid sequence for organisms within the genus *Legionella* are quite similar to those of other genera (i.e., *Pseudomonas* and *Vibrio*). Within the genus *Legionella* the target nucleic acid sequence is not the same for all species. A blend of the two capture probes were made in order to detect most of the organisms of the genus *Legionella* in a single detection testing.

These two capture probes are 18 bases long and vary by one base; one probe has a "G" base where the other has a "A". The Tm of these two probes are approximately 60 and 58 C respectively. A titration was done to choose the proper ration of these capture probes and found to be in this case 1:1. A 1:1 mixture of the probes were made and the mixture was immobilized on a membrane. This mixture capture probe is used for the detection of the genus *Legionella*. This mixture makes it possible to detect most of the species of *Legionella*. If either of the probes were used alone only partial detection of the genus *Legionella* would be possible under stringent conditions. Under reduced stringent conditions, non-*Legionella* organisms with similar 5S rRNA sequences such as organisms of the genus *Pseudomonas* could also be detected and produce false positive results.

It will be apparent to those of skill that various substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention. The following examples are provided for

illustration purposes and should not be construed as a limitation of this invention.

EXAMPLES

A. Isolation of Sample DNA

A water sample of between 100 to 500 ml is first filtered through a 25 mm, .45μ pore filter (Durapore from Millipore). The filter is transferred to a tube containing 2 ml of DNA extraction reagent(20% Chelex® resin, 10 mM Tris-HCl, 0.1 mM EDTA pH 8.0) and vortexed to free the organisms from the filter surface. The sample is placed in a boiling water bath and heated to 99°C in a heat block for 10 min to lyse the organisms.

B. PCR Amplification of the *Legionella* genome

As previously stated, the preferred PCR procedure is a multiplex reaction where both the *mip* and 5S rRNA target regions are amplified simultaneously in the PCR mixture. A 20 μl sample from the above test solution is removed and added to 65 μl of an amplification reaction mix containing the following: PCR buffer (50 mM KCl, 50 mM Tris-HCl at pH 8.9); 4 units of Taq DNA polymerase (from PECI), 0.35 mM dNTPs and 0.5 μM of each primer. The primers are described in table 2. Three hundred copies of the internal positive control (IPC) sequence is added to the reaction mixture. The IPC sequence is provided in Table 3. The sequences extending beyond PT70 are not amplified and can be deleted.

The target 5S rRNA gene subsequence of the *Legionella* genus and the target subsequence of the *mip* gene of *Legionella pneumophila* are amplified using PCR procedures which follow.

Table 2. Primers and Probes for Amplifying and Detecting *Legionella* species.*

Left *mip* primer: PT69:	GCATTGGTGCCGATTTGG; Seq. ID No. 6
Right *mip* primer: PT70:	GCTTTGCCATCAAATCTTTCTGAA; Seq. ID No. 7
Right *mip* primer: PT181:	GTTTTGCCATCAAATCTTTTTGAA; Seq. ID No. 23
Left 5S rRNA primer: PT82:	GGCGACTATAGCGRTTTGGAA; Seq. ID No. 10
Left 5S rRNA primer: PT159:	GGCGACTATAGCGGTGTGGAA; Seq. ID No. 21
Right 5S rRNA primer: PT80:	GCGATGACCTACTTTCRCATGA; Seq. ID No.9
Right 5S rRNA primer: PT157:	GCGATGACCTACTTTCGCATG; Seq. ID No. 22
mip capture probe: PT56:	TTGCTTCCGGATTAACATCT; Seq. ID No. 4
mip capture probe: PT35:	CAAGGCATAGATGTTAATCCGG; Seq. ID No. 2
mip capture probe: PT55:	CATAGCGTCTTGCATGCCTTTAGCC; Seq. ID No. 3**
mip capture probe: PT67:	AACCGAACAGCAAATGAAAGACG; Seq. ID No. 5
5S rRNA capture probe: PT77:	CGCGCCAATGATAGTGTGA; Seq. ID No. 8
5S rRNA capture probe: PT100:	CATCTCGAACTCAGAAGTGAAAC; Seq. ID No. 11
5S rRNA capture probe: PT125:	GCGCCAATGATAGTGTG; Seq. ID No. 24
5S rRNA capture probe: PT127:	GCGCCGATGATAGTGTG; Seq. ID No. 25

* Unless stated otherwise all sequences are presented 5′ to 3′.
** preferred sequences

The solution is overlayed with 75 µl of light mineral oil (Sigma Chemical Co., St. Louis, MO), 15 µl of 25 mM $MgCl_2$ is added and the solution is subjected to the following thermal profile using a 1 second setting to change temperatures as rapidly as possible using the Perkin-Elmer Cetus DNA Thermocycler:

1 second to 95°C
95°C for 1 Min.
30 cycles of:
1 second to 95°C
95°C for 1 Min.
1 second to 63°C
63°C for 1.5 Min. followed by
72°C for 7 min and shutdown or hold at 6°C.

Table 3. Internal Positive Control Sequence (IPC) PT74 and Capture Probes.

Seq. ID No. 1

5'-GCATTGGTGCCGATTTGGGGAAGTTTGATGGAGATGAGGAGTTCTACG
****PT69*********
....1.2, 1.3, 4.....>

TGGACCTGGAGAGGAAGGAGACTGCCTGGCGGTGGCCTGAGTTCAGCA
..
...1 probe

AATTTGGAGGTTTTGTTCAGAAAGATTTGATGGCAAAGCGTACTGCTGAATTCA-3'
.....
3'*****PT70*************** 5'

Positive Control - DQα1

5'-TGAGTTCAGCAAATTTGGAG-3'; Seq. ID No. 16

Negative Control - DQα 1.2, 1.3, 4

*
5'-GATGAGCAGTTCTACGTGG-3'; Seq. ID No. 17

* represents a single mismatched base pair.

C. Detection of Amplified DNA

Ten microliters of the PCR reaction mixture and 2 μl of a loading gel are mixed and applied to each lane of a 3.0% Nusieve® and 1% Seakem® brand agarose gel (FMC Corp., Rockland, ME) using TBE electrophoresis buffer (89 mM, Tris HCl, 89 mM, sodium borate and 1 mM EDTA at pH 8.2. The loading buffer is 250 mg/ml Ficol 400, 0.25 mg/ml Bromophenol Blue. The gel is 20 cm long and 12.5 cm wide. It is run horizontally for approximately 2 hours at 150 volts. The contents of the gel are then viewed under UV light after ethidium bromide staining.

The sensitivity of this assay permits the detection of 100 copies of the target regions per 10 μl sample. Using primers PT69, PT70 and PT181, the amplified *mip* region is approximately 168 bp and the amplified 5S rRNA region is approximately 107 bp. Under the above conditions the amount of amplified products are approximately equal.

Alternatively the amplified products can be captured on a nylon membrane using capture probes as illustrated in

tables 2 and 3. A mixture of probes PT125 and PT127 gives superior hybridization results when testing for unknown strains of *Legionella*. The probes are bound to a Pall Biodyne-B nylon membrane (Pall Biosupport Div., East Hills, NY).

In a preferred embodiment mixtures represented by PT82 and PT80 are amplified. Eliminating PT 159 and 157 decreases slightly the specificity of the assay for *L. spiritensis, sainthelensi (SG1 and SG2)*, and *quinlivanii*. However the assay is now substantially decreases the undesired amplification of rRNA from *Pseudomonas sps*. More specifically the nonspecific crossreaction is reduced so that 100 copies of a *Legionella* specific target nucleic acid will equal 10^5 to 10^7 *cross-reacting Pseudomonas* specific target.

The capture probes are optionally combined with p-[2-hydroxy-1-naphthyl azo]-benzenesulfonic acid, Orange-11 dye (certified), from Sigma chemical Co., St. Louis, Missouri. The dye is diluted to 0.01% in 50 mM 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS) adjusted to pH 10.0 using sodium hydroxide.

Nylon membrane is cut into strips having a combination of capture probes PT125 and PT127 for 5S RNA, PT55 for the *mip* gene and the positive and negative control probes depicted in table 3. Approximately 4 picomoles of each probe are bound to the membrane in discrete spots. The membrane is then washed with a mixture of 0.5% SDS w/v, 5X SSPE (diluted from 20X standard saline phosphate EDTA buffer pH 7.4 as prepared in Sambrook, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989) and 0.03% dextran sulfate.

Forty μl of denaturation solution (1.2 N NaOH, 0.11M EDTA) is added to the exemplified sample. A 50 μl sample of the denatured sample is added to approximately 3 milliliters of hybridization solution comprising 0.5% w/v SDS in 5X SSPE. The nylon membranes with capture probes bound thereto are each exposed to 3 ml of hybridization solution in a water bath under gentle rotation at 50-55°C for 20 min.

The hybridization solution is decanted from the membranes and biotinylated amplified target bound to the

membrane is linked to horseradish peroxidase through streptavidin. The enzyme is attached by incubating the membranes under gentle rotation in a 50-55°C water bath for 20 minutes in 3.0 ml of 2.5X SSPE with 0.10% w/v SDS and 25 µl of a commercially available streptavidin-horseradish peroxidase solution (AmpliType Kit™ DQα DNA typing kit from Perkin Elmer Cetus). The nylon membrane are washed once for 12 minutes at 50-55°C under gentle rotation in an excess of 2.5X SSPE with 0.1% w/v SDS as wash buffer and finally washed with shaking at room temperature for 5 minutes in 2.5X SSPE with 0.1% SDS and again in 0.1 M sodium citrate at pH 5.0.

Color development is achieved by using 3,3',5,5' tetramethylbenzidine (TmB). 0.25 ml of a TMB stock solution of 2mg/ml in ethanol is diluted in 5 ml of 0.5 M sodium citrate at pH 5.0 and 1 µl of a standard solution of hydrogen peroxide. The membranes are incubated at room temperature for 30 minutes in the presence of this solution in the dark. The membranes are then rinsed twice in 10 ml of water at room temperature and results are recorded.

WHAT IS CLAIMED IS:

1. Nucleic acid polymerase primers for the amplification of subsequences of nucleic acid from *Legionella* species wherein the primers bind substantially to the nucleic acid subsequence selected from the group consisting of

(a) 3'-CGTAACCACGGCTAAACC-5', Seq. ID No. 12;
(b) 3'-CGAAACGGTAGTTTAGAAAGACTT-5', Seq. ID No. 13;
(c) 3'-CCGCTGATATCGCYAAACCTT-5', Seq. ID No. 14;
(d) 3'-CGCTACTGGATGAAAGYGTACT-5', Seq. ID No. 15;
(e) 3'-CCGCTGATATCGCCACACCTT-5', Seq. ID No. 18;
(f) 3'-CGCTACTGGATGAAAGCGTAC-5', Seq. ID No. 19; and
(g) 3'-CAAAACGGTAGTTTAGAAAAACTT-5', Seq. ID No. 20

where Y represents a cytosine or thymine base and the primers having a base Y encompass a mixture of the two primers.

2. Nucleic acid polymerase primers of claim 1 wherein the primers bind substantially to the nucleic acid subsequence selected from the group consisting of:

(e) 3'-CCGCTGATATCGCCACACCTT-5', Seq. ID No. 18;
(f) 3'-CGCTACTGGATGAAAGCGTAC-5', Seq. ID No. 19; and
(g) 3'-CAAAACGGTAGTTTAGAAAAACTT-5', Seq. ID No. 20.

3. A nucleic acid polymerase primer of claim 1 wherein the primer binds substantially to the nucleic acid subsequence 3'-CGTAACCACGGCTAAACC-5', Seq. ID No. 12.

4. A nucleic acid polymerase primer of claim 1 wherein the primer binds substantially to the nucleic acid subsequence 3'-CGAAACGGTAGTTTAGAAAGACTT-5', Seq. ID No. 13.

5. A nucleic acid polymerase primer of claim 1 wherein the primer binds substantially to the nucleic acid subsequence 3'-CCGCTGATATCGCYAAACCTT-5', Seq. ID No. 14.

6. A nucleic acid polymerase primer of claim 1 wherein the primer binds substantially to the nucleic acid subsequence 3'-CGCTACTGGATGAAAGYGTACT-5', Seq. ID No. 15.

7. A nucleic acid polymerase primer of claim 1 wherein the primer binds substantially to the nucleic acid subsequence 3'-CCGCTGATATCGCCACACCTT-5', Seq. ID No. 18.

8. A nucleic acid polymerase primer of claim 1 wherein the primer binds substantially to the nucleic acid subsequence 3'-CGCTACTGGATGAAAGCGTAC-5', Seq. ID No. 19.

9. A nucleic acid polymerase primer of claim 1 wherein the primer binds substantially to the nucleic acid subsequence 3'-CAAAACGGTAGTTTAGAAAAACTT-5', Seq. ID No. 20.

10. Nucleic acid polymerase primers of claim 1 wherein the primers are selected from the group consisting of

(a) 5'-GCATTGGTGCCGATTTGG-3', Seq. ID No. 6;
(b) 5'-GCTTTGCCATCAAATCTTTCTGAA-3', Seq. ID No. 7;
(c) 5'-GGCGACTATAGCGRTTTGGAA-3', Seq. ID No. 10;
(d) 5'-GCGATGACCTACTTTCRCATGA-3', Seq. ID No. 9;
(e) 5'-GGCGACTATAGCGGTGTGGAA-3', Seq. ID No. 21;
(f) 5'-GCGATGACCTACTTTCGCATG-3', Seq. ID No. 22; and
(g) 5'-GTTTTGCCATCAAATCTTTTTGAA-3', Seq. ID No. 23

where R represents a adenine or a guanine base and the primers having a base R are a mixture of the two primers.

11. Nucleic acid polymerase primers of claim 10 wherein the primers are selected from the group consisting of

5'-GGCGACTATAGCGGTGTGGAA-3', Seq. ID No. 21;
5'-GCGATGACCTACTTTCGCATG-3', Seq. ID No. 22; and
5'-GTTTTGCCATCAAATCTTTTTGAA-3', Seq. ID No. 23.

12. Nucleic acid polymerase primers of claim 10 wherein the primers are biotinylated at the 5' end.

13. A nucleic acid polymerase primer of claim 10 wherein the primer comprises 5'-GCATTGGTGCCGATTTGG-3', Seq. ID No. 6.

14. A nucleic acid polymerase primer of claim 10 wherein the primer comprises 5'-GCTTTGCCATCAAATCTTTCTGAA-3', Seq. ID No. 7.

15. A nucleic acid polymerase primer of claim 10 wherein the primer comprises 5'-GGCGACTATAGCGRTTTGGAA-3', Seq. ID No. 10.

16. A nucleic acid polymerase primer of claim 10 wherein the primer comprises 5'-GCGATGACCTACTTTCRCATGA-3', Seq. ID No. 9.

17. A nucleic acid polymerase primer of claim 10 wherein the primer comprises 5'-GGCGACTATAGCGGTGTGGAA-3', Seq. ID No. 21.

18. A nucleic acid polymerase primer of claim 10 wherein the primer comprises 5'-GCGATGACCTACTTTCGCATG-3', Seq. ID No. 22.

19. A nucleic acid polymerase primer of claim 10 wherein the primer comprises 5'-GTTTTGCCATCAAATCTTTTTGAA-3', Seq. ID No. 23.

20. A nucleic acid polymerase primer of claim 18 wherein the primer is biotinylated at the 5'-end.

21. A nucleic acid polymerase primer of claim 19 wherein the primer is biotinylated at the 5'-end.

22. An internal positive control oligonucleotide sequence comprising an unnatural oligonucleotide subsequence flanked by a nucleic acid subsequence selected from the group consisting of

(a) 3'-CGTAACCACGGCTAAACC-5', Seq. ID No. 12;
(b) 3'-CGAAACGGTAGTTTAGAAAGACTT-5', Seq. ID No. 13;
(c) 3'-CCGCTGATATCGCYAAACCTT-5', Seq. ID No. 14;
(d) 3'-CGCTACTGGATGAAAGYGTACT-5', Seq. ID No. 15;

(e) 3'-CCGCTGATATCGCCACACCTT-5', Seq. ID No. 18;
(f) 3'-CGCTACTGGATGAAAGCGTAC-5', Seq. ID No. 19; and
(g) 3'-CAAAACGGTAGTTTAGAAAAACTT-5', Seq. ID No. 20
where Y represents a cytosine or thymine base.

23. An oligonucleotide sequence of claim 22 comprising a nucleic acid subsequence selected from the group consisting of 3'-CGTAACCACGGCTAAACC-5', Seq. ID No. 12; and, 3'-CGAAACGGTAGTTTAGAAAGACTT-5', Seq. ID No. 13.

24. An oligonucleotide sequence of claim 22 comprising a nucleic acid subsequence selected from the group consisting of 3'-CCGCTGATATCGCYAAACCTT-5', Seq. ID No. 14; and 3'-CGCTACTGGATGAAAGYGTACT-5', Seq. ID No. 15;
where Y represents a cytosine or thymine base.

25. An oligonucleotide sequence of claim 22 comprising a nucleic acid subsequence selected from the group consisting of 3'-CCGCTGATATCGCCACACCTT-5', Seq. ID No. 18; 3'-CGCTACTGGATGAAAGCGTAC-5', Seq. ID No. 19; and 3'-CAAAACGGTAGTTTAGAAAAACTT-5', Seq. ID No. 20.

26. An oligonucleotide sequence of claim 22 comprising a nucleic acid subsequence of 5'-GCATTGGTGCCGATTTGG-3', Seq. ID No. 6.

27. An oligonucleotide sequence of claim 22 comprising a nucleic acid subsequence of 5'-TTCAGAAAGATTTGATGGCAAAGC-3', Seq. ID No. 13.

28. An oligonucleotide capture probe comprising a nucleic acid subsequence of: 5'-CATAGCGTCTTGCATGCCTTTAGCC, Seq. ID No. 3; 5'-GCGCCAATGATAGTGTG-3', Seq. ID No. 24; 5'-GCGCCGATGATAGTGTG-3', Seq. ID No. 25; or mixtures thereof.

29. A process of using nucleotide polymerases for amplification of nucleic acid subsequences from *Legionella* species wherein the polymerases initiate subsequence

amplification by extension of nucleic acid primers which bind substantially to a nucleic acid subsequence selected from the group consisting of:

(a) 3'-CGTAACCACGGCTAAACC-5'; Seq. ID No. 12;
(b) 3'-CGAAACGGTAGTTTAGAAAGACTT-5'; Seq. ID No. 13;
(c) 3'-CCGCTGATATCGCYAAACCTT-5'; Seq. ID No. 14;
(d) 3'-CGCTACTGGATGAAAGYGTACT-5'; Seq. ID No. 15;
(e) 3'-CCGCTGATATCGCCACACCTT-5'; Seq. ID No. 18;
(f) 3'-CGCTACTGGATGAAAGCGTAC-5'; Seq. ID No. 19; and
(g) 3'-CAAAACGGTAGTTTAGAAAAACTT-5'; Seq. ID No. 20

where Y represents a cytosine or thymine base and the primers having a base Y are a mixture of the two primers.

30. A process of claim 22 wherein the primers are selected from the group consisting of:

(a) 5'-GCATTGGTGCCGATTTGG-3', Seq. ID No. 6;
(b) 5'-GCTTTGCCATCAAATCTTTCTGAA-3', Seq. ID No. 7;
(c) 5'-GGCGACTATAGCGRTTTGGAA-3', Seq. ID No. 10;
(d) 5'-GCGATGACCTACTTTCRCATGA-3', Seq. ID No. 9;
(e) 5'-GGCGACTATAGCGGTGTGGAA-3', Seq. ID No. 21;
(f) 5'-GCGATGACCTACTTTCGCATG-3', Seq. ID No. 22; and
(g) 5'-GTTTTGCCATCAAATCTTTTTGAA-3', Seq. ID No. 23.

31. A process of claim 22 which further comprises co-amplifying with the nucleic acid subsequences from *Legionella* species, an internal positive control oligonucleotide sequence flanked by upstream and downstream sequences which are complementary to at least one of the primers used to amplify the *Legionella* species.

32. A kit for the amplification of nucleic acid from *Legionella* species comprising a compartment which contains a nucleic acid which binds substantially to a nucleic acid subsequence selected from the group consisting of:

(a) 3'-CGTAACCACGGCTAAACC-5', Seq. ID No. 12;
(b) 3'-CGAAACGGTAGTTTAGAAAGACTT-5', Seq. ID No. 13;
(c) 3'-CCGCTGATATCGCYAAACCTT-5', Seq. ID No. 14;
(d) 3'-CGCTACTGGATGAAAGYGTACT-5', Seq. ID No. 15;

(e) 3'-CCGCTGATATCGCCACACCTT-5', Seq. ID No. 18;
(f) 3'-CGCTACTGGATGAAAGCGTAC-5', Seq. ID No. 19; and
(g) 3'-CAAAACGGTAGTTTAGAAAAACTT-5', Seq. ID No. 20
where Y represents a cytosine or thymine base and the primers having a base Y are a mixture of the two primers.

33. A kit of claim 32 wherein the nucleic acid is selected from the group consisting of:
(a) 5'-GCATTGGTGCCGATTTGG-3', Seq. ID No. 6;
(b) 5'-GCTTTGCCATCAAATCTTTCTGAA-3', Seq. ID No. 7;
(c) 5'-GGCGACTATAGCGRTTTGGAA-3', Seq. ID No. 10;
(d) 5'-GCGATGACCTACTTTCRCATGA-3', Seq. ID No. 9;
(e) 5'-GGCGACTATAGCGGTGTGGAA-3', Seq. ID No. 21;
(f) 5'-GCGATGACCTACTTTCGCATG-3', Seq. ID No. 22; and
(g) 5'-GTTTTGCCATCAAATCTTTTTGAA-3', Seq. ID No. 23
where R represents a adenine or a guanine base and the primers having a base R are a mixture of the two primers.

34. A kit of claim 31 which further comprises an internal control oligonucleotide sequence flanked by upstream and downstream sequences which are complementary to the primers used to amplify the *Legionella* species.

35. A method for controlling visual density of detection probes bound to capture probes in a nucleic acid hybridization assay having first and second capture probes wherein the nucleic acid sequences of the two capture probes are different, said method comprising:
(a) mixing a blend of the two capture probes;
(b) affixing the mixture of probes from step (a) to a first discrete region of a solid support and the first capture probe to a second discrete region of a solid support wherein the concentration of the first capture probe is equal in both regions and the concentration of the second capture probe in the second region is different from the concentration of second capture probe in the first region;
(c) binding the detection probes to the capture probes of step (b) under high stringent hybridization

conditions such that the detection probes bind to one capture probe but not the other; and

(d) detecting hybridization of the detection probe by visual density;

wherein the proportion of the two capture probes affixed to the first region is adjusted to provide visual density equal to that of the second region when detection probes are present in equal amounts and are permitted to bind under the hybridization conditions of step (c).

36. A method of claim 35 wherein the detection probe is an amplification product.

37. A method of claim 36 wherein the detection probe acid is a polymerase chain reaction amplification product.

38. A method of claim 35 wherein the detection probe is a complex of a target nucleic acid which binds under high stringent hybridization conditions to different regions of the capture and signal probe and wherein the capture and signal probes do not hybridize to each other.

39. A method of claim 38 wherein the target nucleic acid is an amplification product.

40. A method of claim 39 wherein the target nucleic acid is a polymerase chain reaction amplification product.

41. A method of claim 35 wherein the proportion of the two capture probes in the blend of step (a) is adjusted according to a known quantity of target nucleic acid.

42. A method of claim 35 wherein the detection range for the capture probe is in a concentration range of between about 10×10^{-12} to about 100×10^{-9} molar.

43. A method of claim 36 wherein the assay provides a positive gradient of visual density over a target nucleic

acid concentration of between about 10 x 10^{-12} to about 100 x 10^{-9} molar.

44. A nucleic acid hybridization kit providing equal visual density for positive results in a test sample, said kit using detection probes, and first and second capture probes wherein the capture probes have different nucleic acid sequences, said kit comprising the following components:

(a) a solid support having a first discrete region having a concentration of the first and second capture probes affixed thereto;

(b) a solid support having a second discrete region having the first capture probes affixed thereto in a concentration equal to the concentration of the first probe in the first region of component (a) and the concentration of the second probe is not equal to the concentration of second probe in the first region;

(c) a container containing detection probes;

wherein the proportion of the two capture probes in the mixture of component (a) is adjusted to give a visual density equal to that of the solid support of component (b) under identical and high stringent hybridization conditions when equal amounts of the detection probes are present in the test sample.

45. A kit of claim 44 further comprising a container containing amplification primers for amplifying the detection probe.

46. A kit of claim 45 further comprising a container containing a DNA polymerase.

47. A kit of claim 44 wherein the detection probe comprises a target nucleic acid which binds to different regions of the capture and signal probe and wherein the capture and signal probes cannot hybridize to each other.

48. A kit of claim 47 further comprising a container containing DNA polymerase to amplify the target nucleic acid.

49. A kit of claim 44 wherein the proportion of the two capture probes in the blend of component (a) is adjusted to provide a uniform density when a known quantity of detection probe is bound to the probes.

50. A kit of claim 44 wherein the detection range for the detection probe is within a concentration range of between about 10×10^{-12} to about 100×10^{-9} molar.

51. A kit of claim 50 wherein the assay provides positive gradient of visual density for the detection probe over a concentration of between about 10×10^{-12} to about 100×10^{-9} molar.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/09688

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): C07H 15/12, 17/00; C12P 19/34; C12Q 1/70
US CL : 435/6, 91; 536/ 27, 28, 29; 935/ 8, 76, 77, 78

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	435/6, 91; 536/27, 28, 29; 935/8, 76, 77, 78

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
Y	US, A, 4,683,195 (Mullis et al.) 28 July 1987, see column 19, line 41-column 20, line 2.	1-34
Y	WO, A, 88/03957 (HOGAN ET AL.) 02 June 1988, see abstract.	1-34
Y	Journal of Clinical Microbiology, Volumn 27, Number 6, issued June 1989, Starnbach et al., "Species-Specific Detection of Legionella pneumophila in Water by DNA Amplification and Hybridization," pages 1257-1261, see abstract.	1-34
Y	FEMS Microbiology Letters, Volume 65, issued 1989, Bottger, "Rapid determination of bacterial ribosomal RNA sequences by direct sequencing of enzymatically amplified DNA," pages 171-176, see entire document.	1-34
Y	Nucleic Acids Research, Volume 15, Number 3, issued 1987, MacDonell et al. "The nucleotide sequence of the 5S rRNA from Legionella pneumophila," page 1335, see entire document.	1-34

* Special categories of cited documents:[15]

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
13 MARCH 1992	19 MAR 1992
International Searching Authority[1]	**Signature of Authorized Officer[20]**
ISA/US	L. Yuan

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	Krieg et al., "Bergy's Manual of Systematic Bacteriology, Volume 1" published 1984 by Williams and Wilkins (Baltimore), see pages 8-11, especially page 9.	1-34
Y	Infection and Immunity, Volume 57, Number 4, issued April 1989, Engleberg et al., "DNA Sequence of mip, a *Legionella pneumophila* Gene Associated with Macrophage Infectivity," pages 1263-1270, see abstract.	1-34

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _, because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. ☐ Claim numbers _, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. ☐ Claim numbers _, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
Y	Applied and Environmental Microbiology, Volume 54, Number 9, issued September 1988, Steffan et al., "DNA Amplification to Enhance Detection of Genetically Engineered Bacteria in Environmental Samples," pages 2185-2191, see abstract.	1-34
Y	Chemical Abstracts, Volume 112, Number 25, Chemical Abstracts Accession number CA112(25):232270x, Adler et al., "Nucleic acid amplification and hybridization method for rapid nucleic acid detection," WO 89-10970, 16 November 1989.	35-51